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CELLULAR AND MOLECULAR IMMUNOLOGY

SECOND EDITION

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these cells become quiescent, develop into memory cells, or differentiate into end-cells with short half-lives.

c. Antigens and the immune responses to these antigens stimulate a number of mechanisms whose principal function is feedback regulation of the response itself. These regulatory mechanisms are discussed in Chapter 10.

5. *Discrimination of self from non-self.* One of the most remarkable properties of the immune system is its ability to distinguish between foreign antigens and self antigens. Thus, the lymphocytes in each individual are able to recognize and respond to many foreign antigens but are normally unresponsive to the potentially antigenic substances present in that individual. Immunologic unresponsiveness is also called **tolerance.** Self-tolerance is an acquired process that has to be learned by the lymphocytes of each individual. It occurs in part because lymphocytes pass through a stage in their development when encounter with antigen leads to their death or inactivation. Thus, potentially self-recognizing lymphocytes come into contact with self antigens at this stage of functional immaturity and are prevented from developing to a stage at which they would be able to respond to self antigens. A great deal is now known about the selection processes that are responsible for self-tolerance, and these will be discussed in Chapters 8 and 19. Abnormalities in the induction or maintenance of self-tolerance lead to immune responses against self (autologous) antigens, and debilitating diseases that are called **autoimmune diseases.** The generation and pathologic consequences of autoimmunity are described in Chapter 19.

These five cardinal features of specific immunity are necessary if the immune system is to perform its normal function of host defense. Specificity and memory enable the immune system to mount heightened responses to persistent or recurring stimulation with the same antigen and thus to combat infections that are prolonged or occur repeatedly. Diversity is essential if the immune system is to defend individuals against the many potential pathogens in the environment. Self-limitation allows the system to return to a state of rest after it eliminates each foreign antigen, thus enabling it to respond optimally to other antigens that the individual encounters. Self-tolerance and the ability to distinguish between self and non-self are vital for preventing reactions against one's own cells and tissues while maintaining a diverse repertoire of lymphocytes specific for foreign antigens.

PHASES OF IMMUNE RESPONSES

All immune responses are initiated by the recognition of foreign antigens. This leads to activation of the lymphocytes that specifically recognize the antigen, and culminates in the development of mechanisms that mediate the physiologic effect of the response, namely elimination of the antigen. Thus, specific immune responses may be divided into (1) the **cognitive phase,** (2) the **activation phase,** and (3) the **effector phase** (Fig. 1–3). Throughout this book, we will discuss the mechanisms of specific immunity in the context of these three phases.

Cognitive Phase

The cognitive phase of immune responses consists of the binding of foreign antigens to specific receptors on mature lymphocytes that exist prior to antigenic stimulation. B lymphocytes, the cells of humoral immunity, express antibody molecules on their surfaces that can bind foreign proteins, polysaccharides, or lipids in soluble form. T lymphocytes, which are responsible for cell-mediated immunity, express receptors that recognize only short peptide sequences in protein antigens. Moreover, T lymphocytes have the unique property of recognizing and responding only to peptide antigens that are present on the surfaces of other cells. The structural basis of antigen recognition by T cells and its physiologic implications are discussed in Chapter 6.

Activation Phase

The activation phase of immune responses is the sequence of events induced in lymphocytes as a consequence of specific antigen recognition. All lymphocytes undergo two major changes in response to antigens. First, they proliferate, leading to expansion of the clones of antigen-specific lymphocytes and amplification of the protective response. Second, lymphocytes differentiate from cells whose primary function is cognitive to cells that function to eliminate foreign antigens. Thus, antigen-recognizing B lymphocytes differentiate into antibody-secreting cells, and the secreted antibody binds the soluble (extracellular) antigen and triggers the mechanisms that eliminate the antigen. Some T lymphocytes differentiate into cells that activate phagocytes to kill intracellular microbes, and other T lymphocytes directly lyse cells that are producing foreign antigens such as viral proteins. The ability of T cells to recognize cell-bound antigens focuses T cell responses in such a way that cell-mediated immunity is effective against intracellular microbes. A general feature of lymphocyte activation is that it usually requires two types of signals: the first is provided by the antigen, and the second by other cells, which may be **"helper cells"** or **"accessory cells."** The nature of these stimuli and the sequence of T and B cell activation are discussed in Chapters 7 and 9.

Two aspects of lymphocyte activation are important in order to allow the small number of cells that respond to any one antigen to perform the many functions that lead to elimination of the antigen. First, immunization and antigen recognition trigger numerous amplification mechanisms that rapidly expand the specifically responding cells and, to a lesser extent, bystander cells as well. Second, lymphocytes preferentially migrate to sites of antigen administration and immune responses. The cellular and biochemical mechanisms of amplification and lymphocyte migration are discussed in later chapters.

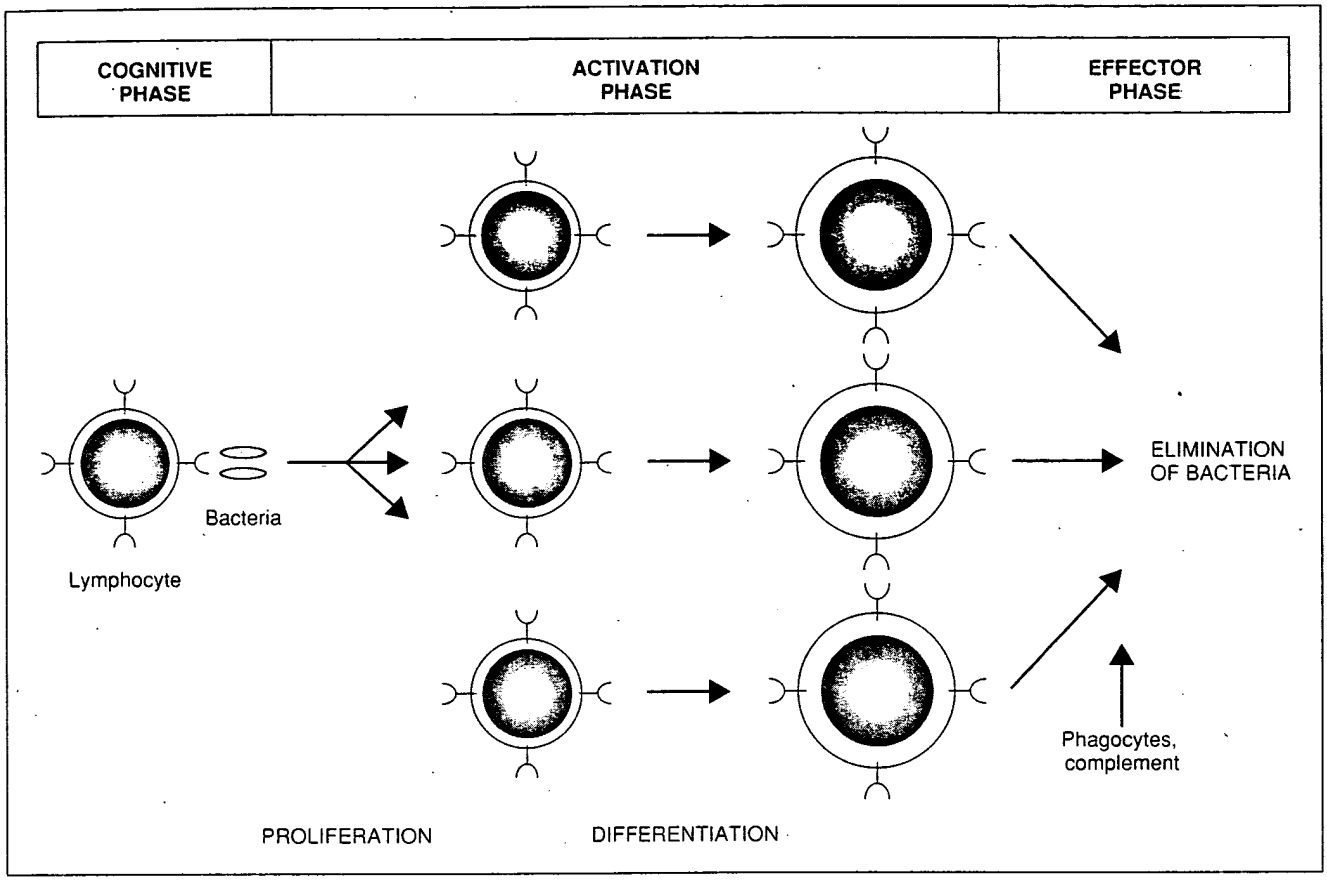

FIGURE 1–3. Phases of specific immune responses. *Immune responses consist of three phases:* cognitive *(antigen recognition),* activation *(proliferation and differentiation of lymphocytes), and* effector *(elimination of antigen). This example illustrates an immune response to bacteria, but the same phases are seen in all specific immune responses. Since this applies to both B and T lymphocytes, the lymphocytes shown can be of either class.*

Effector Phase

The effector phase of immune responses is the stage at which lymphocytes that have been specifically activated by antigens perform the functions that lead to elimination of the antigen. Lymphocytes that function in the effector phase of immune responses are called **effector cells.** Many effector functions require the participation of other, non-lymphoid cells (which are also often referred to as "effector cells") and defense mechanisms that are also operative in natural immunity. For instance, antibodies bind to foreign antigens and enhance their phagocytosis by blood neutrophils and mononuclear phagocytes. Antibodies also activate a system of plasma proteins termed **complement,** which participates in the lysis and phagocytosis of microbes (see Chapter 15). Other antibodies stimulate the degranulation of mast cells and the release of mediators, which combat infections and are responsible for the vascular components of acute inflammation (see Chapter 14). Activated T lymphocytes secrete protein hormones, called **cytokines,** which enhance the functions of phagocytes and stimulate inflammatory responses (see Chapters 12 and 13). Phagocytes, complement, mast cells, cytokines, and the leukocytes that mediate inflammation are all components of natural immunity, because they do not specifically recognize or distinguish between different foreign antigens, and they are all involved in defense against microbes, even without specific immune responses. Thus, the effector phase of specific immunity illustrates a fundamental concept that was emphasized earlier in this chapter—that specific immune responses serve to amplify and focus onto foreign antigens a variety of effector mechanisms that are also functional in the absence of lymphocyte activation (Fig. 1–4).

THE CLONAL SELECTION HYPOTHESIS

From the initial demonstration that the immune system could respond specifically to a vast number of foreign antigens, the problem of explaining how such a diverse repertoire could be generated and maintained was appreciated by immunologists. Two mutually exclusive hypotheses were proposed to explain the specificity and diversity of immune responses, even before there was a clear understanding of the importance of

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Review

Jaks, STATs, Cytokine Signal Transduction, and Immunoregulation: Are We There Yet?

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The impatient refrain "Are we there yet?," echoing from the back seat, is a familiar one to any parent. The question, though, is a valid one for the impatient immunologist struggling to understand the regulation of the immune response. That is, abundant data exist to substantiate the role of various cytokines in immunoregulation (Paul and Seder, 1994; Abbas et al., 1996). Concomitantly, striking advances have been made recently in understanding cytokine signaling. But do these advances provide a satisfactory molecular explanation for cytokine actions and the processes involved in regulating the immune response?

The discoveries of Janus kinases (Jaks) (Figures 1 and 2) and signal transducers and activators of transcription (STATs) (Figure 3) have explained a great deal about signaling by cytokine receptors. One appeal of the Jak/STAT pathway is that the trail from membrane to gene regulation is remarkably direct. In addition, although the Jaks do not provide an explanation for the specificity of cytokine signaling, cytokine receptors and STATs do. This review focuses on the relationship of cytokine signaling to immunoregulation. In particular, humans and mice that lack specific Jaks and STATs are discussed (Table 1), because these examples provide clear illustrations of the importance of the Jak/STAT pathway in controlling the immune response.

Cytokines, Cytokine Receptors, and Immunoregulation

The term "cytokine" encompasses an array of diverse soluble factors. One subset of cytokines includes more than 30 factors and comprises the α-helical cytokines. These cytokines bind to a class of receptors known as type I cytokine receptors and include interleukins, colony-stimulating factors, and hormones (Bazan, 1990). Closely related are the receptors for the interferons (type II cytokine receptors). This superfamily can be further divided into subgroups based on the use of shared subunits (Taga and Kishimoto, 1995; Leonard, 1996). For instance, the common γ chain (γ_c) is a subunit of the interleukin-2 (IL-2), IL-4, IL-7, IL-9, and IL-15 receptors. This feature of cytokine receptors provides one molecular explanation for the redundant nature of cytokines.

The importance of cytokines in immunoregulation is now well documented. IL-12 and interferon-γ (IFNγ) are important in promoting cell-mediated responses, and IL-12 knockout mice have impaired T helper cell 1 (Th1) responses (Magram et al., 1996). Conversely, IL-4 and IL-5 are key mediators of allergic responses, and IL-4 knockout mice have impaired Th2 responses (Kuhn et al., 1991; Kopf et al., 1993). IL-7 is important for the development and/or survival of T cells and B cells; mice lacking this cytokine or its receptor are profoundly lymphopenic (von Freeden-Jeffry et al., 1995; Peschon et al., 1994). IL-2, however, has both positive and negative effects on the immune response. A major abnormality seen in IL-2–, IL-2 receptor α chain (IL-2Rα)–, and IL-2Rβ–deficient mice is the development of autoimmune disease and lymphoid expansion (Kundig et al., 1993; Sadlack et al., 1993; Willerford et al., 1995; Suzuki et al., 1995). IL-10 is another cytokine that appears to hold the immune response in check; IL-10 knockout mice also have severe immunologic disease (Kuhn et al., 1993). Thus, it is clear that cytokines play a central role in immunoregulation. But how does this occur? What does this mean on a molecular level? How do specific cytokines regulate the expression of specific genes?

Cytokine Signal Transduction and Jaks

Unlike growth factor receptors and the transforming growth factor β/activin family of receptors, which have intrinsic enzymatic activity (as tyrosine and serine/threonine kinases, respectively), cytokine receptors lack intrinsic catalytic activity. Rather, they are associated with a structurally unique class of kinases, the Jaks: Jak1, Jak2, Jak3, and Tyk2 (Figures 1 and 2) (reviewed by Ihle et al., 1995; Johnston et al., 1996). The function of Jaks was uncovered by the generation of cell lines resistant to the effects of interferons, whose defects could be complemented by expression of different Jaks (Velazquez et al., 1992; Muller et al., 1993a; Silvennoinen et al., 1993; Watling et al., 1993). Subsequently, it was found that various Jaks are activated by all the α-helical cytokines (summarized by Ihle, 1995; Johnston et al.,

	Chromosome	
	Human	Mouse
Jak 1	1p31.1	4
Jak 2	9p24	19
Jak 3	19p13.1	8
Tyk 2	19p13.2	
Hop	*Drosophila*	

Figure 1. Structure of Jaks

Among metazoan tyrosine kinases, the Jaks are structurally unique in that they contain a C-terminal catalytic domain and an adjacent pseudokinase domain. These segments constitute the first two Jak homology (JH) domains, JH1 and JH2, respectively. Other conserved segments within the Jaks have been noted and are identified as JH domains, although their functions have not been ascertained. However, the N terminus has been found to be important in the binding of the Jaks to cytokine receptors. The chromosomal localization of the Jaks is also shown. See Ihle et al., 1995.

1996). Jak1 and Jak2 are activated by a broad range of cytokines, and Tyk2 is activated by cytokines that utilize gp130 (IL-6 and other cytokines), IL-10, IL-12, and IL-13 in addition to IFNα/β. In contrast, Jak3 specifically associates only with γ_c (Russell et al., 1994; Miyazaki et al., 1994; Boussiotis et al., 1994) and is only activated by cytokines that bind to γ_c-containing receptors. The use of Jaks by different cytokine receptors is illustrated in Figure 4.

Thus, Jaks fit the bill as key mediators of signaling by cytokine receptors in that they physically associate with cytokine receptor subunits and are essential for cytokine signaling.

Jaks and Development

What is the significance to the organism of the absence of a given Jak? Thus far we only have information on one mammalian Jak, Jak3. Mutation of γ_c, which specifically associates with Jak3, is the molecular basis of X-linked severe combined immunodeficiency (X-SCID) (Table 1) (Noguchi et al., 1993; Leonard, 1996). The function of γ_c as a component of many cytokine receptors helps to explain the severity of this immunodeficiency. The intimate association of Jak3 and γ_c suggested that mutations of Jak3 itself might also cause SCID (Russell et al., 1994), and patients with autosomal recessive SCID due to Jak3 mutations were subsequently identified

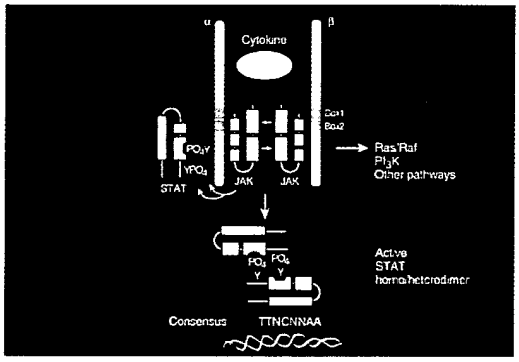


Figure 2. A Model for the Role of Jaks and STATs in Cytokine Signal Transduction

Cytokine binding to receptor subunits induces homo- or heterodimerization resulting in the apposition of Jaks that are bound to the receptor chains. The N terminus of the Jaks is probably important for receptor association. Bringing the Jaks into proximity allows the Jaks to become activated, likely through transphosphorylation. Like other tyrosine kinases, phosphorylation of tyrosine residues within the activation loop of the kinase domain is probably an essential part of this activation. The activated Jaks phosphorylate the cytokine receptor subunits providing docking sites for proteins with SH2 domains. The STAT family of transcription factors is one important class. STATs bound to cytokine receptors are themselves phosphorylated on a C-terminal tyrosine residue. This site in turn is recognized by the SH2 domain of another STAT molecule allowing dimerization to occur. Homo- and hetero-dimerized STATs translocate to the nucleus and bind DNA, thereby regulating gene expression. Other mechanisms may be involved in STAT–receptor interactions and alternative means for STAT activation may exist. Aside from their role as transcription factors, STATs may also function as adapter molecules for coupling receptors to other signaling pathways. That is, STAT3 has been reported to bind phosphatidylinositol 3-kinase.

(Macchi et al., 1995; Russell et al., 1995). Jak3 knockout mice have also been generated, and they too are immunodeficient (Thomis et al., 1995; Nosaka et al., 1995; Park et al., 1995). Curiously, the phenotype of Jak3-deficiency differs in humans and mice. Whereas Jak3- and γ_c- deficient humans lack T cells but contain B cells (albeit dysfunctional B cells), Jak3- and γ_c-deficient mice (Cao et al., 1995; DiSanto et al., 1995) have a few functionally impaired T cells but lack B cells. Precisely why Jak3- and γ_c-deficiency blocks normal lymphocyte development is not completely understood, but may relate to absent IL-7 signaling. But why the difference between mice and humans? Perhaps there exist alternative receptors or cytokines that rescue B cell development in a species-specific manner.

T cells that are produced in γ_c- and Jak3-deficient mice have an abnormal phenotype. They appear activated in their expression of high levels of CD44 and low levels of CD62L (Thomis and Berg, 1997; Nakajima et al., 1997; Saijo et al., 1997). In the case of Jak3-deficient mice, impaired negative selection in the thymus has been reported (Saijo et al., 1997), suggesting that their abnormal T cell phenotype may reflect activation of autoreactive clones in the periphery. The phenotype of Jak3 deficiency may also be analogous to the lymphoid expansion seen IL-2Rα nullizygous mice. In addition, the phenotype of Jak3 deficiency may be unrelated to γ_c mediated signaling and may reflect a requirement for Jak3 functioning in other signaling pathways. For example, Jak2 has recently been shown to be involved CD40 signaling (Hanissian and Geha, 1997).

Despite the documented essential role of Jaks in cytokine signaling, only Jak3 knockout mice have been generated. However, the zebrafish Jak1 was recently cloned and was shown to play an important role in early vertebrate development (Conway et al., 1997). It was found to be maternally encoded, stored in unfertilized eggs, and expressed throughout the midblastula stage. Thereafter it rapidly disappears but is reexpressed later. The mRNA is evenly distributed among the cells of blastula-stage embryos, and injection of RNA encoding dominant-negative Jak1 kinases inhibited cell migration; reduced expression of goosecoid, a transcription factor that is expressed in dorsal mesoderm; and interfered with anterior structure formation.

Another system that vividly demonstrates the importance of the Jaks in development is the analysis of the function of the Drosophila Jak (Hou and Perrimon, 1997). Termed Hopscotch (HOP), it is structurally remarkably similar to the mammalian Jaks. It is about 26% identical and 50% similar to Jak2; identity within the catalytic (JH1) domain is even greater. Mutation of the *hop* gene results in marked developmental abnormalities through both maternal and zygotic effects. Absence of maternal and zygotic *hop* results in severe segmentation defects, whereas embryos that have one copy of a paternally derived wild-type allele have less severe defects. Progeny from females homozygous for the weak *hop* allele, hop^{msv1}, have subtle segmentation defects. In embryos with mutation of *hop*, stripe-specific defects in the expression patterns of pair-rule genes (*even-skipped*, *runt*, and *fushi tarazu*) and segment-polarity genes (*engrailed* and *wingless*) occur (Binari and Perrimon, 1994). These

Table 1. Summary of the Cytokines That Activate the Jaks and STATs and the Phenotypes Associated with Their Deficiencies

Molecule	Activated by	Phenotype of Deficiency
JAK1	IFNs	ND*
	γ_c cytokines	
	Many others	
JAK2	Many cytokines	ND
JAK3	γ_c cytokines	Combined immunodeficiency#
TYK2	IFNα/β, IL-10, IL-12, others	ND
STAT1	IFNs, other cytokines and growth factors	Viral susceptibility impaired IFN signaling
STAT2	IFNα/β	ND
STAT3	Many cytokines and other stimuli	Embryonically lethal
STAT4	IL-12, IFNα/β (human)	Impaired IL-12 signaling and Th1 development
STAT5A	Many hormones, interleukins, CSFs, other	Impaired lactation and mammary development
STAT5B	Many hormones, interleukins, CSFs, other	ND
STAT6	IL-4	Impaired IL-4 signaling and Th2 differentiation

The Jak and STAT knockout mice that have been produced are described in the text.
* A mammalian Jak1 knockout has not been reported, but in zebrafish, maternally derived Jak1 appears to be essential for embryogenesis (Conway et al., 1997).
# Cases of Jak3 deficiency in humans have been identified, but no human cases have been identified that lack other Jaks or STATs.
ND, no data.

findings raise a number of important questions. For instance, how many Jaks do Drosophila have, or do they have just one? Are there separate Jak1, Jak2 and Tyk2 orthologs? If so, are they regulated by cytokines? Indeed, if flies have cytokines and Jaks, what about other organisms, such as Caenorhabditis elegans? Interestingly, a C. elegans Jak has been identified in the sequencing of the C. elegans genome. Clearly this is a very old pathway for cellular differentiation.

Jaks and Transformation

In several circumstances, mutations of Jaks provide clear evidence that Jaks are essential for normal growth and development. But what about transformation? Can dysregulation of Jaks lead to cancer? Of great interest in this regard is that activating mutations of *hop* also have striking consequences. These mutations, known as *Tum-l* (tumorous lethal) mutations, result in leukemia in flies (Hanratty and Dearolf, 1993; Harrison et al., 1995; Luo et al., 1995; Luo et al., 1997). Thus far, however, no circumstances have been identified in which a human cancer is the result of mutation or translocation of a Jak. Nonetheless, there are a number of circumstances in which constitutive activation of Jaks is associated with malignant transformation. This was first demonstrated in human T cell leukemia virus I–transformed T cells (Migone et al., 1995). Constitutive Jak activation has also been found in other settings, including Sezary's syndrome (Zhang et al., 1996), v-*abl*–transformed cells (Danial et al., 1995), and acute lymphoblastic leukemia (Meydan et al., 1996). Finally, transformation may also result from mutations of cytokine receptor subunits. Two examples are mutations of the thrombopoietin receptors (v-*mpl*) (Souyri et al., 1990) and erythropoietin receptors (Longmore and Lodish, 1991), both of which lead to constitutive dimerization of the receptors and, hence, constitutive Jak activation.

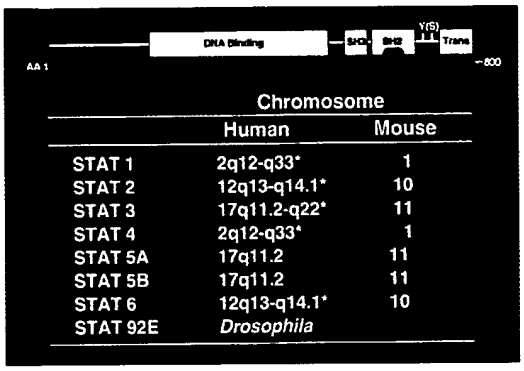
Other Unanswered Questions Pertaining to Jaks

A number important points regarding the Jaks remain incompletely understood. For instance, how does Jak structure related to its functions and how is Jak catalytic activity regulated? Phosphorylation of tyrosine residues that reside in the activation loop of tyrosine kinases typically positively regulate catalytic activity. For Tyk2 and Jak2, mutation of these tyrosines are known to inhibit Jak activity (Gauzzi et al., 1996; Feng et al., 1997). However, there are many other conserved tyrosine residues within the Jaks whose functions are not yet understood. Variant Jak isoforms have also been identified. In the case of Jak3, three splice variants that contain identical N-terminal regions but that diverge at the C termini have been isolated. One form appears to be catalytically inactive, but its precise role in signaling is not understood (Lai et al., 1995).

An intriguing aspect of the structure of Jaks is the presence of a pseudokinase domain, a feature that is conserved among insect, fish, and mammalian Jaks but whose function has not been clearly defined. Mutations in the JH2 domain clearly alter Jak function (Luo et al., 1997; Velazquez et al., 1995), and the presence of this catalytically inactive domain may be important in regulating enzymatic activity. In addition, it has recently been reported that the JH2 domain binds STATs (Fujitani et al., 1997). Thus, it is unlikely that this segment is simply vestigial. Clearly, structural studies of the Jaks are eagerly anticipated.

Our ignorance of the details of Jak structure and function extends to the remaining conserved segments of the Jaks. Jaks have a domain that is reminiscent of a Src homology 2 (SH2) domain, although the ability of this domain to bind phosphorylated tyrosine residues has not been demonstrated. Mutation of the arginine corresponding to the conserved residue in other SH2 domains did not have an identifiable consequence (Kohlhuber et al., 1997).

In a number of receptor systems it has been demonstrated that Jaks are constitutively associated with cytokine receptor subunits. The membrane proximal domain of the cytokine receptors appears to be required for association with Jaks, and the proline-rich box 1 motif appears to be particularly important (Figure 2) (Yan et al., 1996b). The component of the Jaks that binds to the cytokine receptor is less clear. However, several studies



	Chromosome	
	Human	Mouse
STAT 1	2q12-q33*	1
STAT 2	12q13-q14.1*	10
STAT 3	17q11.2-q22*	11
STAT 4	2q12-q33*	1
STAT 5A	17q11.2	11
STAT 5B	17q11.2	11
STAT 6	12q13-q14.1*	10
STAT 92E	*Drosophila*	

Figure 3. Structure of STATs

The signal transducer and activator of transcription (STAT) family of transcription factors are notable structurally by the presence of a central DNA binding domain, an SH3-like domain, and an SH2 domain. C-terminal to the SH2 domain there is a conserved tyrosine residue that is phosphorylated upon cytokine stimulation and that is essential for STAT dimerization. The STAT SH2 domain serves both to bind to the phosphorylated cytokine receptor and to effect STAT dimerization. For some STATs, phosphorylation of a C-terminal serine residue (shown in parentheses) may also important for transcriptional activation. The extreme C terminus is divergent and influences transcriptional activation. The N terminus of the STATs, which is also conserved, is important for protein-protein interactions. Functions ascribed to this region include association with other STATs and receptor binding. The chromosomal localization of the STAT molecules is also shown. The localization of the some STATs (asterisks) is based on mapping of the mouse genes. See Ihle et al., 1995.

have provided evidence that the Jak N terminus is responsible for this binding (Frank et al., 1994; Zhao et al., 1995; Kohlhuber et al., 1997; Chen et al., 1997).

How is Jak signaling terminated? The SH2-containing tyrosine phosphatase, SHP-1, can associate with cytokine receptors (e.g., the erythropoietin receptor) and regulate Jak2 phosphorylation (Klingmuller et al., 1995). Whether other cytokine receptors and Jaks are regulated similarly has not been documented. Jaks are also associated with SHP-2, but is it responsible for Jak dephosphorylation (Yin et al., 1997)? While this has not been determined, SHP-2 generally functions as a positive regulator of signaling and does not attenuate it (Ali et al., 1996).

Although known substrates for Jaks include STATs and receptor chains, there undoubtedly are other Jak substrates as well. For instance, the signal-transducing adapter molecule STAM has been shown to be important in cytokine regulation of the c-*myc* gene and cell growth (Takeshita et al., 1997). Conversely, despite the importance of Jak3 for IL-2 signaling, some aspects of IL-2 signaling may be independent of Jak3. For example, IL-2 induction of Bcl-2 and Bag-1 and phosphorylation of SHP-2 were not inhibited by overexpression of a dominant negative allele of Jak3, even though IL-2–dependent regulation of c-*fos* and c-*myc* was abrogated and cellular proliferation was inhibited (Kawahara et al., 1995; Adachi et al., 1996, 1997). Indentifying other Jak substrates and dissecting Jak-dependent and Jak-independent signaling pathways will be important.

Thus, much work remains to be done to elucidate the structure and function and the regulation of the Jaks. Nevertheless, it is already clear that activation of specific Jaks cannot explain the specificity of cytokine signaling. First, multiple cytokines activate the same Jaks (e.g., IL-2 and IL-4). Second, experimentally recruiting different Jaks to a given cytokine receptor does not affect the specificity of the signal. So, if the Jaks do not provide signal specificity, what does? The answer is that signal specificity resides in the STAT family of tyrosine-phosphorylated transcription factors.

STATs: Their Critical Role in Transmitting Cytokine Signals

STATs were first discovered in protein complexes bound to the promoters of interferon-inducible genes (reviewed by Darnell et al., 1994; Schindler and Darnell, 1995; Ihle, 1996). Seven STATs have now been cloned: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (Figure 3). These transcription factors bind a nucleotide consensus motif of TTNCNNNAA, termed a GAS (IFNγ–

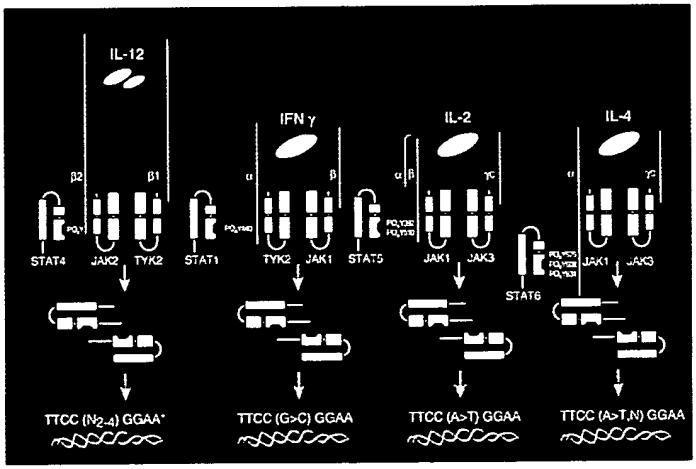


Figure 4. Mechanisms by Which Specificity Is Achieved in Cytokine Signaling

The membrane proximal region of cytokine receptors is responsible for the binding of distinct Jaks likely through their N termini. However, this does not explain how different STATs are activated by different cytokines. Rather, specific residues in the receptor that surround phosphorylated tyrosines are recognized by STAT SH2 domains, thus providing a mechanism for the recruitment of different STATs to different receptors. Although a consensus binding sequence can be defined for STATs, the different STATs bind DNA elements with some selectivity, providing an additional layer of specificity. The optimal STAT4 binding sites are not well defined (asterisks).

activated site) element even though this sequence binds STATs induced by a variety of cytokines (Figure 3). The interferon response element (ISRE) is different in its composition, in that it is a nonpalindromic motif, TTTCNNTTTC, that binds a complex of proteins (ISGF3): STAT1, STAT2, and a non-STAT protein, p48.

It was first shown that IFNα/β activates STAT1 and STAT2 whereas IFNγ activates only STAT1 (Schindler et al., 1992, Fu, 1992; Shuai et al., 1992; Silvennoinen et al., 1993; Shuai et al, 1993). Later it was shown that some STATs, such as STAT3 and STAT5, are activated by a variety of cytokines, whereas others seem to have more limited function. For instance, STAT6 is activated primarily by IL-4 (Hou et al., 1994) (Figure 4); STAT4 in mice is activated only by IL-12, although in humans it is also activated by IFNα/β (Bacon et al., 1995; Jacobson et al., 1995; Cho et al., 1996). The essential function of STAT proteins in cytokine signaling was demonstrated by the observation that IFNα/β signaling requires both STAT1 and STAT2 whereas IFNγ signaling requires STAT1 (Muller et al., 1993; Leung et al., 1995). Notably, STAT1 can be activated by additional cytokines and can even be activated by noncytokine stimuli, such as epidermal growth factor (Sadowski et al., 1993). Nevertheless, mice made deficient in STAT1 by gene targeting have highly specific defects (Durbin et al., 1996; Meraz et al., 1996) (Table 1). STAT1-deficient mice lack interferon signaling and have marked susceptibility to viral infections.

Similarly, the phenotypes of STAT4 and STAT6 knockout mice are also quite discrete (Table 1). STAT4 knockoutmice are viable and fertile and have normal hematopoiesis, but are unable to respond to IL-12: IL-12–induced mitogenesis, enhancement of natural killer cytolytic function, and Th1 differentiation are markedly impaired. In addition, development of Th1 cells in response to challenge with Listeria monocytogenes is abrogated in STAT4-deficient mice, which have a propensity for development of Th2 cells. Thus, STAT4 is essential for mediating responses to IL-12 in lymphocytes and regulates T helper cell differentiation (Kaplan et al., 1996; Thierfelder et al., 1996).

STAT6 knockout mice have deranged IL-4 signaling and cannot generate a Th2 response (Kaplan et al., 1996, Shimoda et al., 1996; Takeda et al., 1996). In STAT6-deficient mice, IL-4–induced up-regulation of major histocompatibility class II expression, CD23, and IL-4 receptor is abrogated. IL-4–mediated proliferation of T and B cells is also reduced. STAT6-deficient B cells do not produce immunoglobulin E (IgE) following immunization in vivo, and STAT6-deficient T lymphocytes do not differentiate into Th2 cells in response to either IL-4 or IL-13. The production of Th2 cytokines from T cells, as well as IgE and IgG1 responses, after nematode infection are also profoundly reduced. Thus, STAT6 is essential for mediating lymphocyte responses to IL-4. In contrast, leptin, a cytokine that regulates obesity, also has been reported to activate STAT6 (Ghilardi et al., 1996). However, STAT6-deficient mice are not obese, suggesting that STAT6 is not an irreplaceable component in leptin signaling.

In contrast to STAT4 and STAT6, which are activated by a limited number of cytokines, STAT5A and STAT5B are activated by an extensive list of cytokines, including prolactin, growth hormone, erythropoietin, thrombopoietin, granulocyte–macrophage colony-stimulating factor, IL-2, and others. Even IFNγ has been shown to activate STAT5 (Meinke et al., 1996). Surprisingly, the defects in STAT5A knockout mice are also remarkably discrete (Liu et al., 1997). STAT5A knockout mice develop normally and are indistinguishable from normal mice in terms of size, weight, and fertility. However, mammary lobuloalveolar outgrowth during pregancy is absent, and females fail to lactate after parturution because of failure of mammary gland differentiation. Although STAT5B has 96% similarity with STAT5A and is expressed coordinately during mammary gland development, STAT5B does not compensate for the absence of STAT5A. Thus, STAT5A is an essential mediator of mammopoietic and lactogenic signaling. A caveat, however, is that STAT5B phosphorylation is reduced in STAT5A gene–targeted mice, for reasons that are presently unclear. Surprisingly, no abnormality of lymphoid of hematopoietic development has been detected in these mice.

STAT3 is activated by many cytokines, including some that activate STAT5A and STAT5B. In sharp contrast to STAT5A knockouts, no viable STAT3-deficient mice have been obtained (Takeda et al., 1997). Analysis of embryos at several gestation times revealed that STAT3-deficient embryos showed degeneration between embryonic days 6.5 and 7.5, indicating that STAT3 is essential for early embryonic development.

Thus, with the exception of STAT3, which has essential functions in embryogenesis, other STATs have limited and specific functions even if they can be activated by diverse stimuli.

A Drosophila STAT (termed Marelle, DSTAT, and STAT92E) has been cloned and underscores the importance of STATs in cellular differentation (Hou and Perrimon, 1997; Hou et al., 1996; Yan et al., 1996c, 1996d). Mutation of the *stat92e* gene exhibits a phenotype identical to that seen with mutations of *hop*. It is important that reduction of *stat92e* gene activity suppresses the phenotype associated with a gain-of-function hop^{tum-l} mutation. Conversely, it also enhances the phenotype associated with a weak *hop* mutation. HOP then activates STAT92E to regulate transcription. As with mammalian STATs, STAT92E is phosphorylated on tyrosine, and the phosphorylated protein binds specifically to a STAT DNA consensus sequence. Furthermore, two STAT-binding sites have been identified within the *eve stripe 3* enhancer region, and mutations in either site greatly decrease *stripe 3* expression in transgenic flies. Therefore, the invertebrate Jak/STAT system is clearly involved in Drosophila early development and is remarkably similar to that of mammals. Several questions arise: How many STATs do insects have? Like vertebrates, do invertebrates have specialized STATs that serve distinct functions, or is there a single STAT? If insects and worms have Jaks and STATs, do they also have cytokines and cytokine receptors? How many different ones do they have, and how do they regulate differentiation? How did the vertebrate immune system pirate this pathway for its own regulation?

Remarkably, a STAT protein, termed Dd-STAT, has

recently been identified in Dictostelium (Kawata et al., 1997). This protein was identified as a transcription factor that bound to the promoter of the *ecmA* gene, which is involved in prestalk cell differentiation. It is homologous to metazoan STATs in the N terminus and DNA-binding domain. Moreover, it has an SH2 domain and is regulated by tyrosine phosphorylation. The element in the *ecmA* gene has some similarity to an ISRE, and Dd-STAT can bind a typical mammalian ISRE. It is notable that this factor functions as both a transcriptional activator and a repressor. The identification of a Dictostelium STAT suggests that STATs probably arose early in the evolution of multicellular organisms to mediate intracellular communication. Although tyrosine kinases cloned from Dictyostelium have a pseudokinase domain upstream from the catalytic domain, they are not closely related to Jaks in other respects (Adler et al., 1996). Thus, STATs may have preceded Jaks evolutionarily.

Whereas STATs have been clearly documented to have essential roles in cellular differentiation, their role in cellular proliferation has been controversial. Mutated versions of receptors that can no longer bind STATs have had variable effects on the ability of receptors to transmit a proliferative signal. Various STATs knockout mice, however, have blunted proliferative responses, indicating that STATs do contribute to cellular proliferation. Studies in Drosophila also indicate a role for STATs in both proliferation and transformation. In mammalian cells there are also circumstances in which constitutive STAT activation is associated with transformation, although the mechanisms are not understood. In some cases of transformation, STAT activation is concomitant with constitutive Jak activation, but in other cases, STAT activation has been found without Jak activation. Some examples of this include transformation with v-*src* (Yu et al., 1995; Cao et al., 1996) and spleen focus-forming virus (Ohashi et al., 1996). Although STAT activation has been found in Bcr/Abl transformation, analysis of Jak activation has yielded conflicting results (Carlesso et al., 1996; Ilaria and Van Etten, 1996; Shuai et al., 1996). Constitutive STAT activation has been reported in human leukemic cells by some groups (Gouilleux-Gruart et al., 1996; Weber-Nordt et al., 1996) but not by others (Kanwar et al., 1996).

Undoubtedly, STAT activation alone, however, does not account for the proliferative response to cytokines; other pathways activated by cytokine receptors contribute to this response. This point is also discussed below.

How STATs Accomplish Specific Signaling from Receptors to Genes

Gene-targeted mice demonstrate that STATs have highly specific functions and are essential for immunoregulation. But how are specific STATs activated by specific cytokines? And how do STATs turn on specific genes? In addition to a central DNA-binding domain (Horvath et al., 1995; Schindler et al., 1995), STATs have a single SH2 domain near their C termini (Figure 2). Like other SH2 domains, the STAT SH2 domain binds to phosphorylated tyrosine residues, with adjacent residues influencing binding specificity. The model that Jaks phosphorylate receptor subunits to create a docking site that recruits a specific STAT is well supported by existing data (Figure 3) (Greenlund et al., 1994; Hou et al., 1994; Heim et al., 1995; Stahl et al., 1995; Yan et al., 1996a). For example, IL-4 stimulates phosphorylation of STAT6, which preferentially binds the sequence ASSGEEGY-PO_4-KPFQDLI, found within the IL-4 receptor α chain (Schindler et al., 1995) (Figure 4). Similarly, IFNγ activates STAT1, which selectively binds peptide TSFGY-PO_4-DKPH, within the IFNγ receptor (Greenlund et al., 1995) but cannot bind the IL-4R–derived phosphopeptide. STATs may also preassociate with cytokine receptors prior to phosphorylation via their N termini (Li et al., 1997). After binding to cytokine receptors, STATs are themselves phosphorylated. They are released from the receptor and then dimerize and translocate to the nucleus, where they bind DNA. The dimerization of STATs (homo- and heterodimerization) is also mediated by phosphotyrosine–SH2 interactions (Shuai et al., 1994; Greenlund et al., 1995). That is, phosphorylation of the conserved C-terminal tyrosine residue (e.g., Y701 in STAT1) is essential, and mutation of this tyrosine phosphorylation site or the conserved arginine residue in the STAT SH2 domain abrogates DNA binding and transactivation. STATs also have an SH3-like domain, but its role has not been determined. While STATs are recruited to cytokine receptors and then phosphorylated, there is also an everexpanding list of noncytokine stimuli that also activate STATs. These receptors include growth factor receptors (receptor tyrosine kinases), G protein–coupled receptors, and immune receptors. The mechanisms by which this process occurs have not been well characterized.

Tyrosine phosphorylation of the STATs is probably not the whole story. Several STATs (STAT1, STAT3, and STAT4) have been shown to be serine phosphorylated in response to cytokine stimulation (Boulton et al., 1995; Wen et al., 1995; Zhang et al., 1995; Cho et al., 1996). Serine phosphorylation of STATs appears to be important in transcriptional regulation, but whether it is essential for DNA binding is less clear. For STAT1, the site of phosphorylation is S727. This residue lies within the divergent C terminus, which appears to be important for transcriptional activation. This site is also a consensus phosphorylation site for a proline-directed serine kinase, such as mitogen-activated protein kinase (MAPK). MAPK has been reported to be activated by interferon and to be associated with the interferon receptor (David et al., 1995). MAPK is activated by other cytokines, but at this time the precise significance of STAT serine phosphorylation and the identity of the STAT serine kinase(s) are controversial. Like STAT1, STAT5 has been shown to be serine phosphorylated (Beadling et al., 1996), but STAT does not have a consensus MAPK phosphorylation site in the C-terminal transcriptional activation domain.

Specific cytokine receptors can recruit specific STATs, but then what? Do STATs have any specificity in terms of the DNA sequences that they bind? The answer appears to be yes (Schindler and Darnell, 1995; Seidel et al., 1995; Ihle, 1996) (Figure 4). Although a core GAS sequence is identifiable, the precise spacing and composition of this motif appear to be distinct among different STATs. For instance, the spacing between the palindromic halfsites that generate an optimal STAT6 binding

site (TTCCNNGGAA) is different from those for other STATs. The delineation of these preferred sites is far from complete, but it is becoming clear how a cytokine such as IL-4 recruits a specific transcription factor that can turn on specific genes. Conversely, the observation that some DNA sequences indiscriminately bind STATs can explain the overlapping biological effects of some cytokines. The regulatory regions for genes such as the IL-12Rβ2 gene will be interesting to dissect, since expression of this gene is positively regulated by IFNγ (in mice) and IFNα/β (in humans) but negatively regulated by IL-4 (Rogge et al., 1997; Szabo et al., 1997). One of the most intriguing recent developments is the finding that the core GAS element is bound not only by STATs but also by another transcription factor, Bcl-6. Even more striking is that Bcl-6 knockout mice have marked cardiac and pulmonary eosinophilic infiltrates. In addition, they have exaggerated production of Th2 cytokines. Bcl-6 can repress IL-4–mediated transcription of a STAT6-activated gene (Dent et al., 1997). One explanation for the pathologic features of Bcl-6–deficient mice is unopposed IL-4 signaling due to the lack of a repressor that binds STAT6 sites. Whether other STAT repressor proteins exist is not known.

It is now well recognized that gene regulation is typically effected by the coordinated binding of multiple transcription factors to the regulatory elemens. Within the IFNγ gene, STAT sites are arranged in multiple copies, and cooperative interactions among N-terminal domains are required for optimal STAT binding (Xu et al., 1996). Such cooperative binding interactions may enable the STAT proteins to recognize variations of consensus sites, promoting selectivity in transcriptional activation. STATs have also been reported to associate with other proteins, including the glucocorticoid receptor and p300/CBP (Bhattacharya et al., 1996; Stocklin et al., 1996). Understanding the regulation of genes by cytokines will require careful dissection of the promoter binding sites of these genes and delineation of the interactions of STATs and other transcription factors.

Conclusions and Future Directions

The discovery of the Jak/STAT pathway goes a long way toward explaining cytokine signaling. The requirement for Jaks in cytokine signaling is very clear, as highlighted by the severe immunodeficiency in humans and mice with Jak3 mutations. Despite their importance for transducing cytokine receptor signals, the Jaks do not seem to contribute to the specificity of cytokine signaling. But STATs do. There are two levels by which specificity of cytokine signaling is achieved: (1) STAT binding to tyrosine- phosphorylated sites on specific cytokine receptors via STAT SH2 domains and (2) STAT binding to specific DNA elements.

How do we proceed from here? One important issue is to define more completely the genes that are regulated by cytokines and to analyze their promoters. Compared with our knowledge about genes induced by interferons, much less is known about the genes induced by IL-2, IL-4, and IL-12. Examining the relative contribution of STATs and other transcription factors on the expression of these genes should provide substantial insights into the mechanisms of cytokine action.

Presently, our understanding of the STAT function is limited. Although they are translocated to the nucleus after activation, we understand neither the mechanism underlying this translocation nor the reason that are they retained in the cytoplasm prior to stimulation. In addition, two mechanisms have been proposed for attenuating STAT activation: ubiquitination and dephosphorylation (Kim and Maniatis, 1996; Haspel et al., 1996). However, the relative importance of these two mechanisms and the identity of the STAT phosphatase are not known. Also, multiple isoforms exist for several STATs, some which function as inhibitors of the full-length forms (Wang et al., 1996). What is the physiologic function of these alternate STAT species, and how is the process regulated? Last, the solution of the three-dimensional structure of STAT proteins will be of great interest and should improve our understanding of how these transcription factors are regulated, how they bind DNA, and how they accomplish specific binding.

Now that a variety of knockout mice have been generated, these mice will provide excellent vehicles for reconstitution studies with mutated version of Jaks and STATs to elucidate structure–function relationships as they relate to the organism. Moreover, given the specific phenotypes of STAT1, STAT4, and STAT6 knockout mice, the search for human mutations of these genes seems warranted. For STAT3, which has global effects on development, it will be important to investigate the loss of such a factor in a tissue- and organ-specific manner to delineate its role in the adult animal.

A particularly important issue is how cytokines regulate cell growth and the contribution of Jaks and STATs to this process. Clearly, other pathways, including the Ras/Raf/MAPK and phosphatidylinositol 3-kinase/AKT pathways are also turned on by cytokine receptors. How do Jaks and STATs interact with this pathway? Recent data show that STATs also serve as adapter molecules for the coupling of cytokine receptors with phosphatidylinositol 3-kinase (Pfeffer et al., 1997). Understanding the intersections and crosstalk in these signal pathways is essential. Conversely, it is also pertinent that STATs may have direct effects on inhibiting proliferation by induction of cyclin-dependent kinase inhibitors, such as p21 WAF1/CIP1 (Chin et al., 1996). This may explain the antiproliferative effects of the interferons.

Finally, utilizing model organisms like C. elegans, Drosophila, Dictyostelium, and zebrafish to analyze the components involved in growth and development will undoubtedly promote advances in understanding mammalian cellular differentiation.

So, an appropriate response to the cries from the back seat is "We are not there yet, but keep your eyes open or you'll miss something exciting."

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Note Added in Proof

STAT5B knockout mice have now been reported: Udy, G.B., Towers, R.P., Snell, R.G., Wilkins, R.J., Park, S.-H., Ram, P.A., Waxman, D.J., and Davey, H.W. (1997). Requirement of STAT5b for sexual dimorphism of body growth rates and liver gene expression. Proc. Natl. Acad. Sci. USA *94*, 7239–7244.